



## The role of nighttime water balance on *Olea europaea* plants subjected to contrasting water regimes

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### ABSTRACT

The climate change scenarios besides foreseeing a severe drought imposition also emphasize the temperature rising in the Mediterranean region, with special prominence at nighttime. Despite the high olive tree tolerance to severe environmental conditions, stomatal nighttime water loss can change plant water relations, and the related consequences and opportunities, especially under water scarcity, must be clarified. A set of 3-year-old potted olive trees were subjected to three cycles of drought, imposed by withholding irrigation, while another group were continuously irrigated. At the end of the latter and more severe drought cycle, daytime gas exchange parameters, water status and membrane integrity was negatively affected by drought imposition. Moreover, the nighttime transpiration rate was far above cuticular water loss, suggesting sustained stomatal aperture during nighttime, leading to substantial water losses, which was higher under drought in the first hours of darkness. The higher nighttime stomatal conductance of droughted plants were related with higher starch concentration in their leaves, a thicker trichome layer and a lower intercellular CO<sub>2</sub> concentration, in a closely association with an inferior nighttime respiration. Still, whole-plant transpiration on droughted plants were much lower than leaf transpiration-based estimates, which is interpreted as compensation by water inputs due to dew deposition on leaves. Although unexpected, the increased of stomatal conductance in the first hours of the night, until a certain level of water deficit intensity, could be linked with potential benefits to the plants.

### 1. Introduction

Drought and high temperature stresses impair several physiological processes, like photosynthesis and water status (Fernández, 2014), and seriously affect membrane stability, increasing the permeability and leakage of ions (Elbasyoni et al., 2017), that may lead to decrease vegetative growth and yield. Moreover, those negative effects can be exacerbated by the projections of climate change in the Mediterranean, which anticipate a general reduction in rainfall and an increase in temperature, the last one more markedly at nighttime (IPCC, 2013). Since nocturnal warming could affect plants in several ways, the nighttime transpiration and respiration are worth considering. However, as far as we know these aspects are been poorly investigated.

Although nighttime stomatal opening is unexpected because there is no opportunity for carbon gain and the need to cool leaves is reduced or absent (Caird et al., 2007), a substantial leaf nighttime stomatal conductance ( $g_{night}$ ) and transpiration ( $E_{night}$ ) was observed in a wide range of species from different functional groups and ecosystems (Dawson et al., 2007; Ogle et al., 2012; Resco de Dios et al., 2015; Snyder et al.,

2003). However, there are several factors that can determine those responses, such as soil water (Caird et al., 2007; Dawson et al., 2007; Howard and Donovan, 2007; Escalona et al., 2013; Zeppel et al., 2014) and nutrient availability (Caird et al., 2007), vapor pressure deficit (VPD) (Daley and Phillips, 2006; Caird et al., 2007; Dawson et al., 2007; Zeppel et al., 2014), wind speed (Daley and Phillips, 2006; Dawson et al., 2007), CO<sub>2</sub> concentration (Caird et al., 2007; Zeppel et al., 2014), dusts, aerosols and dew and/or fog (Burkhardt, 2010), previous day environmental conditions (Caird et al., 2007; Easlon and Richards, 2009), net photosynthesis ( $A_N$ ) (Easlon and Richards, 2009) and stomatal conductance during the day ( $g_{day}$ ) (Snyder et al., 2003), carbohydrate metabolism (Easlon and Richards, 2009; Resco de Dios et al., 2015), circadian rhythms (Caird et al., 2007; Resco de Dios et al., 2015) and leaf age (Caird et al., 2007; Zeppel et al., 2014). The occurrence and magnitude of  $g_{night}$  and  $E_{night}$  display conflicting patterns and the generalization about the factors that affect those traits is still not possible. Additionally, there are strong evidences that the responses are both species and cultivar dependent (Daley and Phillips, 2006; Escalona et al., 2013; Flexas et al., 2010; Ogle et al., 2012; Snyder et al., 2003).

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Although recently we have noticed a growing awareness about  $g_{night}$  and  $E_{night}$ , their implication in physiological processes remain unclear (Coupel-Ledru et al., 2016; Escalona et al., 2013; Ogle et al., 2012). The  $g_{night}$  and, consequently,  $E_{night}$  affect plant water balance, water use efficiency (WUE) (Escalona et al., 2013) and hydraulic redistribution (Howard et al., 2009). In fact, substantial water losses have been reported to occur overnight at leaf and plant scales, strongly impacting global evapotranspiration (Forster, 2014; Resco de Dios et al., 2015). Nevertheless, it has also been postulated several benefits related with the continue water loss during the night that may outweighs those costs, including the improvement of nutrient uptake (Scholz et al., 2007; Snyder et al., 2008; Snyder et al., 2003), preventing excess cell turgor at night when water availability increases and leaves still contain substantial contents of osmoticants (Donovan et al., 1999, 2001), supplying  $O_2$ , enhancing the capacitance of the trunk and stem (Daley and Phillips, 2006) and preventing  $CO_2$  build-up in leaves from nighttime dark respiration ( $R_{night}$ ) (Marks and Lechowicz, 2007). The prevalence of  $g_{night}$  and  $E_{night}$  in some conditions and the potential influence in plant growth and physiology can indicate a widespread behavior and an adaptive process that must be clarified. Furthermore, those outcomes have implications in plant water relations theory and in the studies, that utilize plant water use data at larger scales (Dawson et al., 2007).

Olive tree (*Olea europaea* L.) is a common species of Mediterranean region that displays important morphological and physiological adaptive mechanisms to withstand the environmental constraints that characterizes Mediterranean climate. This capacity, includes the ability to control leaf transpiration by a high thickness and density of the leaves, associated to a dense peltate trichomes layer (Bacelar et al., 2004). Additionally, also involves an efficient capacity to regulate stomatal aperture, and the effective ability to extract water from soils with very low water potential and/or to sustain very low internal water deficits (Connor and Fereres, 2005). However, there is a lack of information and understanding about  $g_{night}$  and  $E_{night}$  mechanisms in *Olea europaea*. Arquero et al. (2006) mentioned a very low stomatal conductance during the night, becoming to rise 3 h before dawn in olive cuttings of cv. Chemali de Sfax. In addition, the study of  $g_{night}$  and  $E_{night}$  by leaf-level gas exchange includes the loss across both cuticular and stomatal components (Caird et al., 2007). This limitation can be avoided by determining the cuticular transpiration ( $E_{cuticular}$ ).

Nocturnal warming is likely to have a significant effect on respiration rate (R) (Catoni et al., 2013; Turnbull et al., 2002), especially on the maintenance component that, in opposite to growth respiration, increases exponentially with temperature (Peraudeau et al., 2015). As plant biomass production depends on the balance between  $A_N$  and R (Pérez-Priego et al., 2014), R is a determining factor to maintain growth and productivity, particularly in conditions in which  $A_N$  is negatively affected, such as under drought conditions (Flexas et al., 2005; Galmes et al., 2007; Ribas-Carbo et al., 2005). Although R usually presents an order of magnitude lower than  $A_N$ , photosynthesis is limited temporally, while R occurs continuously in different plant organs (Flexas et al., 2005; Galmes et al., 2007). Contrarily to the  $A_N$  response to drought, that is well investigated, the respiratory process has been commonly marginalized and the few information available tend to be contradictory. Apart to the trend, also the mechanisms underlying R regulation under drought are still largely unknown and most of the studies assessed R on darkened leaves during the day. However, these responses can differ at night, once may change substrate supply and sink demand compared with normal nocturnal conditions (Ribas-Carbo et al., 2005) and, because daytime and nighttime temperatures differ substantially. Improve the knowledge of the importance of R to plant carbon balance during water stress is the first step to enable prediction and management of crop growth and yields in water-stress-prone areas (Flexas et al., 2005). Moreover, may help to hypothesize more accurately species behavior under new climatic conditions (Varone and Gratani, 2015).

Therefore, we address the following questions: (1) It is  $E_{night}$

substantially higher than  $E_{cuticular}$ ?; (2) If so, can different water regimes determine the occurrence and magnitude of  $g_{night}$  and  $E_{night}$ ?; (3) Can leaf anatomical structure influence nighttime water balance?; (4) How  $R_{night}$  responds to different water regimes? (5) What are the most probable causes of  $g_{night}$  and  $E_{night}$ ?; (6) What are the implications and opportunities of  $g_{night}$  and  $E_{night}$  to olive tree?

## 2. Material and methods

### 2.1. Plant material and growth conditions

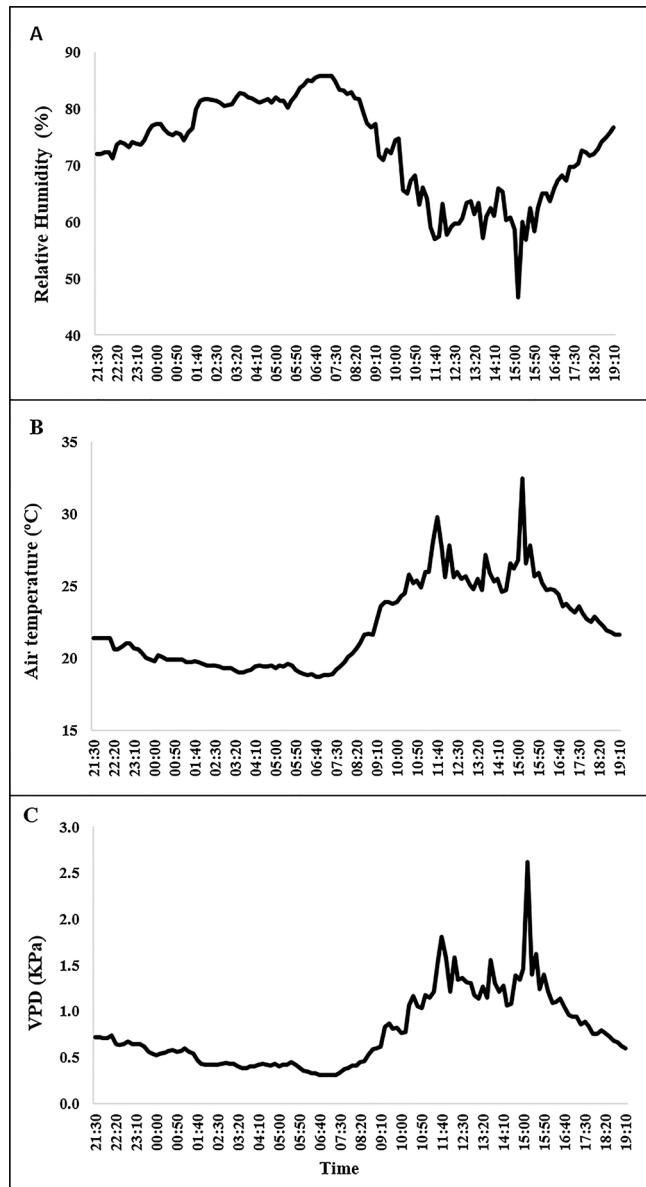
The experiment was carried out with own-rooted 3 years-old olive trees (*Olea europaea* cv. Cobrançosa), between June and September 2014 at the University of Trás-os-Montes and Alto Douro, Vila Real, Northeast Portugal (41°17'17.83"N, 7°44'12.81"W, 448 m a.s.l.). Plants were grown outdoors in 16 l pots containing a mix of sandy-loam soil and horticultural substrate Siro Oliva (Siro-Leal & Soares SA, Mira, Portugal) (2:1). The surfaces of containers were covered with a thin layer of perlite and sealed with plastic film and aluminum foil. This measure aimed to avoid the evaporation from soil surface and the rain water entering to the pots, and to minimize the temperature increase inside the containers. Pots were randomly arranged and periodically rotated to the neighboring position to minimize the effects of environmental heterogeneity. When applicable, plants were watered to field capacity, determined gravimetrically. Care was taken to ensure negligible leaching through the bottoms of the containers during irrigation. All the plants were manually defruited immediately after fruit set to avoid yield influences on the measured variables.

The climate of the study site is typically Mediterranean-like, a warm-temperate climate with dry and hot summers, classified as Csb according to Köppen-Geiger's classification. Mean annual rainfall is 1023 mm, most of which falls in the autumn-winter with negligible rainfall during the summer months, although 2014 was an atypical summer with some rainfall events. The warmest months are July/August and the coldest months are December/January, with mean daily temperatures of 21.3/21.7 °C and 6.8/6.3 °C, respectively (IPMA, 2017). The climatic conditions during the field measurements are presented in Fig. 1.

### 2.2. Experimental plan

Forty uniform selected plants, based on height, leaf number and leaf area were submitted to an acclimatization period of 30 days. At the beginning of the experiment, 6th July, eight plants randomly chosen were harvested to assess the initial biomass of the different plant organs. The remaining thirty-two plants were divided in two groups, each one comprising sixteen plants. One group was kept under well-watered conditions (WW, control plants) throughout the entire experimental period, in which plants were watered every day. The other group was subjected to three "drought-re-watering cycles" (WS, stressed plants) by withholding water until the occurrence of precipitation (1st and 2nd cycles), or until the stomatal conductance for water vapor ( $g_{day}$ ) during mid-morning (peak of photosynthetic activity) dropped around 50  $mmol\ m^{-2}\ s^{-1}$  (reached at 3rd cycle), a threshold value indicating a situation of severe drought stress experienced by the plants; at that value photosynthetic activity becomes predominantly inhibited by metabolic processes, besides stomatal limitations (Flexas and Medrano, 2002). When occurred precipitation, or when olive trees reached the desired drought intensity, they were re-watered to field capacity in the evening and also during the following days until  $A_N$  was almost restored to control values (recovery). The 1st, 2nd and 3rd "drought-re-watering cycles" had the duration of 12–6 days, 9–3 days and 21–16 days, respectively.

Each group of sixteen plants was divided in two subgroups, each one with eight plants arranged in a completely randomized design with four replications (two plants per experimental unit). Plants from one



**Fig. 1.** Relative humidity (A), air temperature (B) and ambient vapor-pressure deficit (VPD) (C) corresponding to the whole-day of physiological measurements.

subgroup were used for physiological destructive measurements, and plants from the other subgroup were used for night-time non-destructive measurements and final biomass assessment. A schematic representation of the experiment is presented in Fig. 2.

All the measurements detailed below were performed 8 times per treatment ( $n = 8$ ), one per plant. Physiological, structural and biochemical measurements at leaf level, measured in healthy, full expanded mature leaves, and whole-plant transpiration records, were performed 21 days after starting the 3rd drought cycle, at the peak of stress. Final biomass accumulation was evaluated at the end of the experiment, 16 days after starting the 3rd re-watering period.

### 2.3. Leaf gas exchange, cuticular transpiration and total plant water balance

During daylight, leaf gas exchange measurements were performed in two periods, morning (mo, 10:00 local time) and midday (mid, 13:30 local time) of a summer cloudless day using a portable IRGA (LCpro +,

ADC, Hoddesdon, UK), operating in the open mode. At night, the measurements were performed in the first hours of the night (22:30–23:30 local time). Just before gas exchange records were taken, dew water was removed with an absorbent paper to avoid interferences in gas exchange measurements. The measurements were made at photosynthetic photon flux density of  $1618 \pm 80$ ,  $1783 \pm 75$  and  $0 \mu\text{mol m}^{-2} \text{s}^{-1}$ , air temperature in the chamber of  $25.0 \pm 0.4$ ,  $30.7 \pm 0.9$  and  $23.1 \pm 0.3^\circ\text{C}$ , and  $\text{CO}_2$  concentration of  $391 \pm 7$ ,  $380 \pm 10$  and  $387 \pm 6 \mu\text{mol CO}_2 \text{ mol}^{-1}$ , at morning, midday and night, respectively. The air flow rate in all periods was set at  $250 \text{ ml air min}^{-1}$ . Net photosynthetic rate ( $A_N$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ), stomatal conductance during the daylight ( $g_{\text{day}}$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ ) and at night ( $g_{\text{night}}$ ,  $\text{mmol H}_2\text{O m}^{-2} \text{s}^{-1}$ ), transpiration rate during the daylight ( $E_{\text{day}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ) and at night ( $E_{\text{night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ), respiration rate at night ( $R_{\text{night}}$ ,  $\mu\text{mol CO}_2 \text{ m}^{-2} \text{s}^{-1}$ ), and the concentration of  $\text{CO}_2$  in intercellular spaces at night ( $C_{\text{inight}}$ ) were estimated using the equations developed by von Caemmerer and Farquhar (1981). Whole-nighttime transpiration ( $E_{\text{w-night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{n}^{-1}$ ) was extrapolated from the gas exchange measurements, assuming constant transpiration during the night, to compare it with the whole-plant water balance estimated by mass losses. Intrinsic water use efficiency was calculated as the ratio of  $A_N/g_{\text{day}}$  ( $\mu\text{mol mol}^{-1}$ ).

In order to discern whether measured values of  $g_{\text{night}}$  and  $E_{\text{night}}$  with IRGA were mostly cuticular or stomatal, cuticular water loss ( $E_{\text{cuticular}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ) was estimated by the weight loss method, as described by Howard and Donovan (2007). Total plant water balance was accessed based on the gravimetric method. Pot mass changes were monitored during a period of 21 h, at early night (22:30 local time), at sunrise (5:30 local time) and at 19:30 (local time) of the following day (balance capacity of 30 kg at 1 g of precision, KERN FKB30K1A, KERN & Sohn GmbH, Balingen, Germany). Plant leaf area was measured at the end of the experiment using the WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK). From pot mass changes and leaf area data were estimated the whole-nighttime transpiration ( $PE_{\text{w-night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{n}^{-1}$ ) and the whole-daytime transpiration ( $PE_{\text{w-daytime}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{daytime}^{-1}$ ).

### 2.4. Leaf water status, structural traits and chemical composition

After the midday gas exchange measurements, leaves were detached and immediately placed into air-tight containers and then the following parameters were examined: fresh weight (FW, g); weight at full turgor (TW, g), measured after immersion of leaf petioles in demineralized water for 48 h in the dark at  $4^\circ\text{C}$ ; leaf area ( $LA$ ,  $\text{cm}^2$ ), measured using the WinDias image analysis system (Delta-T Devices Ltd., Cambridge, UK); and dry weight (DW, g), measured after drying at  $70^\circ\text{C}$  to a constant weight. Further, was calculated the relative water content (RWC) as  $RWC (\%) = (FW - DW)/(TW - DW) \times 100$ , to characterize leaf water status, and the sclerophyll index, leaf mass area (LMA) as  $LMA (\text{g m}^{-2}) = DW/LA$ .

Leaves with the same characteristics, as those used for leaf gas exchange, were collected at the end of the experiment for anatomical tissue measurements. Leaf sections were taken from the middle of the leaves, to avoid differential thickness along the leaf. Cut sections were dehydrated, cleared and embedded in paraffin. Cross-sections of  $4 \mu\text{m}$  were obtained using a rotary microtome, placed on slides and stained with toluidine blue. The thickness of total lamina, palisade and spongy parenchyma, upper and lower epidermis, cuticle and trichome layer were measured in the leaf cross-sections using an inverted optical microscope (Olympus IX51 with the image analysis software Cell'A).

The leaves adjacent to those used for RWC and LMA evaluation, with the same characteristics, were sampled for quantification of non-structural carbohydrates by spectrophotometry. Total soluble sugars (SS) were extracted according to Irigoyen et al. (1992), by heating foliar discs in 80% ethanol during 1 h, at  $80^\circ\text{C}$ . SS were quantified, at 625 nm, after the reaction of the alcoholic extract with fresh anthrone in a

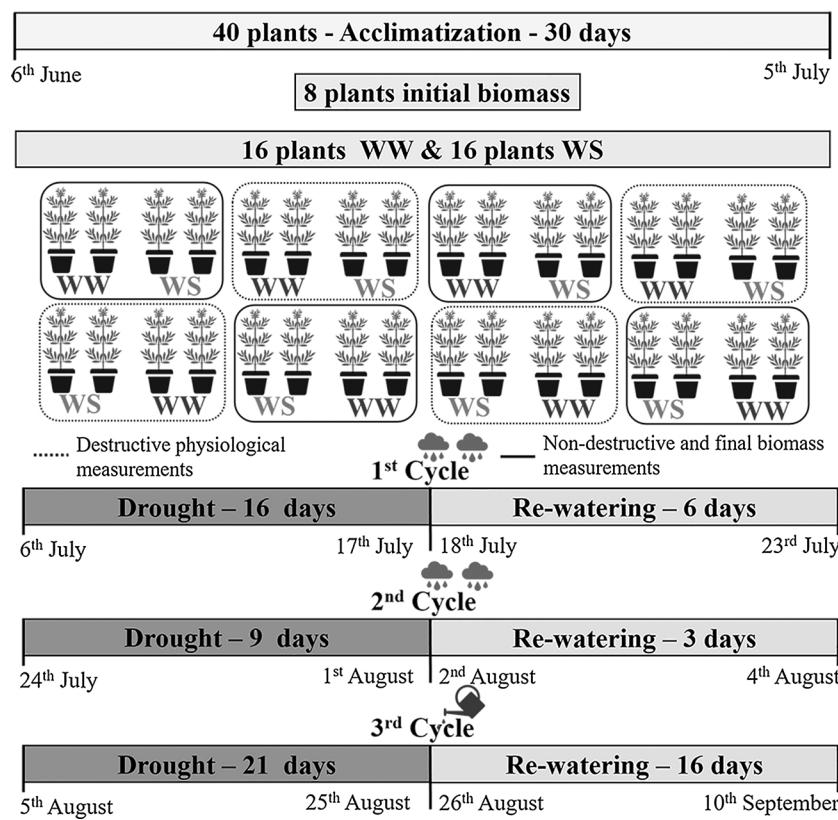


Fig. 2. Schematization of the experiment. Abbreviations: WW, control plants; WS, stressed plants.

boiling water bath for 10 min. Thereafter, starch (St) was extracted from the same solid fraction by heating leaf discs in 30% perchloric acid during 1 h, at 60 °C, according to Osaki et al. (1991). The St concentration was determined by the anthrone method, as described above. Glucose was used as a standard for both SS and St quantification.

### 2.5. Electrolyte leakage

Leaf electrolyte leakage was measured as an indicator of cell membrane permeability, following a procedure described by Mena-Petite et al. (2001) with some modifications. Leaves were washed three times in deionized water to remove surface ions, and then foliar discs (5) of 0.8 cm diameter were punched out of each leaf per plant, and placed in 10 ml deionized water within capped test tubes and incubated for 24 h, at 25 °C, on a rotary shaker. Electrical conductivity of the solution was measured after 24 h. Finally, samples were killed by autoclaving at 120 °C for 20 min, and the total conductivity reading was obtained upon equilibration at 25 °C. The 24 h conductivity was expressed as the percentage of the total conductivity value, having first subtracted the known conductivity value of the deionized water from both values.

### 2.6. Biomass accumulation and whole-plant water use efficiency

At the end of the experiment, the plants of each treatment were harvested and the dry weight of aboveground and belowground organs, after drying in a force-draft oven at 70 °C to a constant weight, were determined.

Water use efficiency of biomass production ( $\text{WUE}_{\text{WP}}$ ) was determined for each plant by dividing total dry matter production by the cumulative amount of water used throughout the growing season, as previously described. Total dry matter included the oven-dried leaves, stems and roots.

### 2.7. Statistical analyses

All statistical calculations were performed using the statistical software program SPSS for Windows (v. 22). After testing for ANOVA assumptions (homogeneity of variances with the Levene's mean test, and normality with the Kolmogorov-Smirnov test), statistical differences were evaluated by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's test ( $P < 0.05$ ). The relationships  $R_{\text{night}}$  and  $SS$ ,  $C_{\text{night}}$  and  $R_{\text{night}}$ ,  $R_{\text{night}}$  and  $E_{\text{night}}$ ,  $g_{\text{night}}$  and  $C_{\text{night}}$ ,  $g_{\text{night}}$  and  $St$ ,  $E_{\text{night}}$  and  $g_{\text{night}}$ ,  $g_{\text{night}}$  and  $g_{\text{mos}}$ ,  $g_{\text{night}}$  and  $g_{\text{mid}}$ ,  $g_{\text{night}}$  and  $A_{\text{mo}}$  and  $g_{\text{night}}$  and  $A_{\text{mid}}$  was analyzed by the Pearson correlation test and significance was set at  $P < 0.05$ .

## 3. Results and discussion

### 3.1. Influence of $E_{\text{cuticular}}$ on $E_{\text{night}}$ , nighttime water balance and responses to water availability

The present study revealed that  $E_{\text{night}}$  and  $g_{\text{night}}$  are important factors that affect whole-plant water balance and water use efficiency in olive tree. The instantaneous  $E_{\text{night}}$  were 2.7–5.9 times higher than the cuticular water losses ( $E_{\text{cuticular}}$ ) in WW and WS plants, respectively (Table 1). The very strong positive correlation ( $r = 0.987$ ;  $P < 0.001$ ) between  $E_{\text{night}}$  (Table 1) and  $g_{\text{night}}$  (Table 2) suggests that most nighttime water losses can be regulated (Howard and Donovan, 2007). Similar evidences of high values of  $g_{\text{night}}$ , which were far above the measured cuticular transpiration, were reported by Howard and Donovan (2007) in *Helianthus* species and by Escalona et al. (2013) in *Vitis vinifera*. In addition,  $E_{\text{cuticular}}$  showed a significant response to water availability, being lower in WS plants, in a strictly association with the significant decrease of RWC from 94 to 64.7% (Table 3). The low cuticular water permeability is one of the main factors supporting the survival and viability of plants under scarce water supply, particularly in species exposed to pronounced levels and rapid changes in

**Table 1**

Water losses of olive control plants (WW) and olive stressed plants (WS), based on cuticular losses ( $E_{\text{cuticular}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ), gas exchange measurements (nighttime transpiration,  $E_{\text{night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ; whole-nighttime transpiration,  $E_{\text{w-night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{n}^{-1}$ ; transpiration at morning period,  $E_{\text{day-mo}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ; transpiration at midday period,  $E_{\text{day-mid}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{h}^{-1}$ ), and mass changes measurements (whole-nighttime transpiration,  $PE_{\text{w-night}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{n}^{-1}$ , and whole-daytime transpiration,  $PE_{\text{w-day}}$ ,  $\text{g H}_2\text{O m}^{-2} \text{daytime}^{-1}$ ). Values are means  $\pm$  SE ( $n = 8$ ). Significant differences: \*\* – significant at  $p < 0.01$ ; \*\*\* – significant at  $p < 0.001$ .

	$E_{\text{cuticular}}$	$E_{\text{night}}$	$E_{\text{w-night}}$	$E_{\text{day-mo}}$	$E_{\text{day-mid}}$	$PE_{\text{w-night}}$	$PE_{\text{w-daytime}}$
WW	4.22 $\pm$ 0.13	11.2 $\pm$ 2.0	84.4 $\pm$ 6.6	372.2 $\pm$ 29.5	296.7 $\pm$ 12.5	177.2 $\pm$ 8.9	1042.0 $\pm$ 72.6
WS	2.82 $\pm$ 0.22	16.5 $\pm$ 1.4	123.9 $\pm$ 6.1	109.5 $\pm$ 9.7	66.8 $\pm$ 6.7	70.6 $\pm$ 13.0	278.9 $\pm$ 36.8
Sig.	**	**	**	***	***	***	***

**Table 2**

Leaf physiological parameters of olive control plants (WW) and olive stressed plants (WS).  $A_{\text{Nmo}}$ ,  $A_{\text{Nmld}}$  represent leaf morning and midday net photosynthetic rates, respectively;  $g_{\text{day-mo}}$ ,  $g_{\text{day-mid}}$ ,  $g_{\text{night}}$  represent leaf morning, midday and night stomatal conductance's, respectively;  $A_{\text{Nmo}}/g_{\text{day-mo}}$ ,  $A_{\text{Nmld}}/g_{\text{day-mid}}$  represent leaf morning and midday intrinsic water use efficiency, respectively;  $R_{\text{night}}$  represent respiration during the night;  $C_{\text{i}}$  represent night intercellular  $\text{CO}_2$  concentration; and  $C_{\text{i}}/\text{Ca}_{\text{mo}}$ ,  $C_{\text{i}}/\text{Ca}_{\text{mid}}$  represent the ratio of intracellular/atmospheric  $\text{CO}_2$  in morning and midday, respectively. Values are means  $\pm$  SE ( $n = 8$ ). Significant differences: \* – significant at  $p < 0.05$ ; \*\* – significant at  $p < 0.01$ ; \*\*\* – significant at  $p < 0.001$ ; n.s. – not significant at  $p > 0.05$ .

Leaf physiological parameters	WW	WS	Sig.
$A_{\text{Nmo}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	18.0 $\pm$ 1.5	6.89 $\pm$ 0.72	**
$A_{\text{Nmld}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	16.2 $\pm$ 0.96	2.93 $\pm$ 0.65	***
$g_{\text{day-mo}}$ ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	172.7 $\pm$ 15.7	53.4 $\pm$ 4.6	***
$g_{\text{day-mid}}$ ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	222.1 $\pm$ 17.4	26.9 $\pm$ 2.4	***
$g_{\text{night}}$ ( $\text{mmol m}^{-2} \text{s}^{-1}$ )	12.2 $\pm$ 0.98	16.5 $\pm$ 1.1	*
$A_{\text{Nmo}}/g_{\text{day-mo}}$ ( $\mu\text{mol mol}^{-1}$ )	102.5 $\pm$ 2.5	131.9 $\pm$ 25.2	n.s.
$A_{\text{Nmld}}/g_{\text{day-mid}}$ ( $\mu\text{mol mol}^{-1}$ )	72.5 $\pm$ 3.5	104.0 $\pm$ 15.2	*
$R_{\text{night}}$ ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ )	2.53 $\pm$ 0.33	1.12 $\pm$ 0.33	*
$C_{\text{i}}$	698.9 $\pm$ 35.6	481.3 $\pm$ 24.3	**
$C_{\text{i}}/\text{Ca}_{\text{mo}}$	0.466 $\pm$ 0.012	0.399 $\pm$ 0.102	n.s.
$C_{\text{i}}/\text{Ca}_{\text{mid}}$	0.608 $\pm$ 0.014	0.490 $\pm$ 0.067	n.s.

**Table 3**

Leaf relative water content (RWC), leaf mass area (LMA), leaf soluble sugars (SS) and starch (St) concentrations, leaf electrolyte leakage (EL) and plant biomass increase (BI) of olive control plants (WW) and olive stressed plants (WS). Values are means  $\pm$  SE ( $n = 8$ ). Significant differences: \* – significant at  $p < 0.05$ ; \*\* – significant at  $p < 0.01$ ; \*\*\* – significant at  $p < 0.001$ ; n.s. – not significant at  $p > 0.05$ .

	WW	WS	Sig.
RWC (%)	94.0 $\pm$ 0.8	64.7 $\pm$ 1.2	***
LMA ( $\text{g m}^{-2}$ )	209.0 $\pm$ 3.0	215.4 $\pm$ 10.6	n.s.
SS ( $\text{mg g}^{-1} \text{FW}$ )	104.6 $\pm$ 6.0	112.7 $\pm$ 4.1	n.s.
St ( $\text{mg g}^{-1} \text{FW}$ )	21.8 $\pm$ 3.6	33.7 $\pm$ 3.0	*
EL (%)	18.0 $\pm$ 1.3	25.7 $\pm$ 0.5	**
BI (%)	62.9 $\pm$ 6.0	24.9 $\pm$ 5.5	**

temperature, as olive tree under the Mediterranean region, where the thermal stability of the cuticular transpiration barrier is decisive, maybe even to a higher degree than the baseline cuticular permeability (Schuster et al., 2016). In our study, the lower  $E_{\text{cuticular}}$  of water stressed plants could be related with differences in cuticle structure and composition, or both, since no differences were observed in cuticle layer thickness (Table 4).

Curiously, WS plants had higher  $g_{\text{night}}$  and, thus,  $E_{\text{night}}$ , during the first hours of darkness (Tables 1 and 2), against the typical evidences that  $g_{\text{night}}$  are most likely to occur when water availability is high (Dawson et al., 2007; Escalona et al., 2013; Flexas et al., 2010; Fuentes et al., 2014; Howard and Donovan, 2007). Nonetheless, the occurrence of  $g_{\text{night}}$  in water-limited habitats was already reported in other species (Ogle et al., 2012; Snyder et al., 2003), as well the higher nocturnal sap flow during the dry season over the wet season (Forster, 2014), strongly

**Table 4**

Leaf tissues thickness ( $\mu\text{m}$ ) (total section, TS; upper cuticle, UC; upper epidermis, UE; upper palisade parenchyma, UPP; spongy parenchyma, SP; lower palisade parenchyma, LPP; lower epidermis, LE; trichome layer, TL) of olive control plants (WW) and olive stressed plants (WS). Values are means  $\pm$  SE ( $n = 8$ ). Significant differences: \* – significant at  $p < 0.05$  and n.s. – not significant at  $p > 0.05$ .

	WW	WS	Sig.
TS	485.7 $\pm$ 11.2	478.3 $\pm$ 8.7	n.s.
UC	7.03 $\pm$ 0.26	7.03 $\pm$ 0.23	n.s.
UE	16.4 $\pm$ 0.8	15.7 $\pm$ 0.5	n.s.
UPP	163.4 $\pm$ 6.1	161.1 $\pm$ 9.1	n.s.
SP	216.5 $\pm$ 5.3	206.9 $\pm$ 3.6	n.s.
LPP	28.6 $\pm$ 1.7	27.6 $\pm$ 1.0	n.s.
LE	15.6 $\pm$ 0.4	15.3 $\pm$ 0.3	n.s.
TL	38.3 $\pm$ 1.5	44.7 $\pm$ 2.0	*

suggesting that this behavior is species dependent, and that may provide an ecological advantage to this sclerophyllous species under most stressful conditions.

Conversely, when nighttime transpiration was estimated based on the gravimetric method (PE), we observed an opposite trend relatively to transpiration extrapolated from  $E_{\text{night}}$  (Table 1). In WW plants,  $E_{\text{w-night}}$  was much lower than  $PE_{\text{w-night}}$ , suggesting a shift on the  $g_{\text{night}}$  throughout the nighttime and/or that gas exchange measurements may underestimate the water losses. Progressive increases of  $g_{\text{night}}$  from early night hours to dawn were reported previously by Escalona et al. (2013), mainly due to a decrease of VPD, and also by some internal regulation of  $g_{\text{night}}$  by sub-stomatal  $\text{CO}_2$  concentration. Our environmental data support these hypothesis, as VPD and temperature decreased toward the dawn (Fig. 1), and the latter may contribute to reduce respiration rate and  $C_i$  values. On the other hand, errors when extrapolating  $E_{\text{night}}$  to the whole-plant are likely to contribute also to such discrepancy, since different leaves, namely the younger leaves, may display significantly different  $E_{\text{night}}$  values and the conditions inside the gas exchange cuvette might not fully reflect the actual conditions of the leaves, leading to biased estimates of  $E_{\text{night}}$  (Escalona et al., 2013).

Noteworthy, in opposite to WW plants,  $E_{\text{w-night}}$  of WS plants was 75% higher than  $PE_{\text{w-night}}$  (Table 1). Similar trend was already described in *Vitis vinifera* by Escalona et al. (2013). Plant water losses can be partially or fully compensated by dew deposition on leaves. It is remarkable the thicker trichome layer of WS plants (Table 4) and the typical (confirmed visually) paraheliotropism response of this species under drought conditions (Bacelar et al., 2009), that could largely increase this dew deposition over the leaves and contribute to the referred discrepancy. In fact, during the night of water balance measurements the dew deposition was confirmed visually, favored by the high relative humidity (72–86%) and the low and decreasing  $\text{VPD}_{\text{ambient}}$  over the night (0.72–0.31 kPa). Additionally, the condensation can start at lower values of relative humidity if the plant dispose of pubescent leaf surfaces that promotes capillary condensation and higher accumulation of hygroscopic particles, as aerosols. This phenomenon takes special importance in arid climates, where it is quite difficult to reach high humidity during the drought season (Burkhardt and Hunsche, 2013;

Konrad et al., 2015). Meanwhile, dew in a dense trichome layer is able to improve WUE indirectly, even without being absorbed by the plant (Konrad et al., 2015), although there are evidences that trichomes play a key role in dew absorption by leaves (Munné-Bosch et al., 1999; Savé et al., 2000). Interestingly, although in WS plants the relative nighttime water losses were slightly more significant than in WW plants, reaching 20.2% against 14.5%, respectively, of the whole-day transpiration (Table 1), the difference was lower than we expected, possibly related with the dew deposition. These results strengthen the need, when is possible, to incorporate both gas exchange and gravimetric measurements in the study of plant nighttime water balance, once extrapolating transpiration from leaf gas exchange measurements to whole-plant transpiration or vice-versa could be erratic.

### 3.2. Photosynthesis and respiration responses to water availability

Net CO<sub>2</sub> assimilation rate of olive trees generally declines in response to severe water deficit (Bacelar et al., 2007a; Ben Abdallah et al., 2018) as in the present study, due to both stomatal and non-stomatal limitations, being the latter more evident at midday period, judging by the changes in  $g_{\text{day}}$ ,  $A_N/g_{\text{day}}$  and Ci/Ca data (Table 2). On the other hand, the trend of R is still not so clear (Varone and Gratani, 2015). It was suggested that R response varies generally from inhibition, with low to moderate stress, to stimulation, with severe stress, being the response closely linked to the species drought tolerance (Atkin and Macherel, 2009; Flexas et al., 2005; Varone and Gratani, 2015). Moreover, it has been assumed that this different response is strongly related with RWC (Flexas et al., 2005; Varone and Gratani, 2015), with 50% being indicated as a threshold value to induce an increase in R (Flexas et al., 2005). This assumption is consistent with our results, since WS plants, with lower  $R_{\text{night}}$  (Table 2), presented RWC of 64.7% (Table 3).

Respiration and net photosynthesis are strongly coupled and intrinsically interdependent because  $A_N$  provides photosynthetic substrates to R, and R supplies ATP and carbon skeletons to sustain plant energy requiring processes (Cannell and Thornley, 2000). Although  $A_N$  declined as a consequence of water deficit, the decrease in  $R_{\text{night}}$  was not related to a decay in the concentration of SS (Table 3). To reinforce this idea, Pearson's correlation between  $R_{\text{night}}$  and SS ( $r = -0.168$ ;  $P = .534$ ) shows that the probability of these variables being correlated is highly unlikely. Similar results were found by Rodríguez-Calcerrada et al. (2011), who suggested that, apart the effect of drought on respiratory capacity, drought-induced reduction of plant growth could translate into a reduction of R in fully developed leaves, via reduced sucrose loading into the phloem and ATP demand. Meanwhile, WS plants accumulate more St in leaves than WW plants (Table 3). Under drought conditions, is expected an increase in SS in spite of St, once drought is known to induce St degradation, resulting in an increase in SS (Chaves, 1991). However, an increase in leaf St content in drought stressed olive trees was already documented (Bacelar et al., 2006), possibly because carbon was not translocated out of the leaves, as these plants were sink-limited as confirmed by lower accumulation of biomass (Table 3), and due to a reduced necessity to increment the use of reserves to increase the maintenance component of R to support extra repair costs (Atkin and Macherel, 2009; Varone and Gratani, 2015). Some studies associate R to other leaf traits, such as leaf tissues thickness and leaf mass per unit of leaf area (LMA) (Lewis et al., 2011; Pérez-Priego et al., 2014; Wright et al., 2006). However, we did not observe this trend on the present study (Tables 2 and 3), probably because sclerophyllly does not change substantially through time in mature leaves (Bacelar et al., 2004), especially in the present growth conditions, with 2 periods of recovery before the 3rd cycle of drought stress. Moreover, Varone and Gratani (2015) argued that higher membrane injury requires an energetic input to the maintenance of the ion gradient across membranes, resulting in higher R. However, although WS plants exhibited higher electrolyte leakage (EL) (Table 3), indicating a

drought-induced membrane impairment, it was not recorded an increase in  $R_{\text{night}}$ . These data confirm that olive tree is highly drought tolerant, since after several days of water shortage the plants are not yet under extreme conditions, being able to reduce the metabolism and conserve the photosynthates. The capacity to maintain low  $R_{\text{night}}$  rates during stressful conditions, associated with a decline in  $A_N$ , allows this species to allocate more assimilates for biomass accumulation and, consequently, for growth (Varone and Gratani, 2015). This could be an important advantage from an evolutionary point of view for plants inhabiting drought-prone and climatic change susceptible habitats.

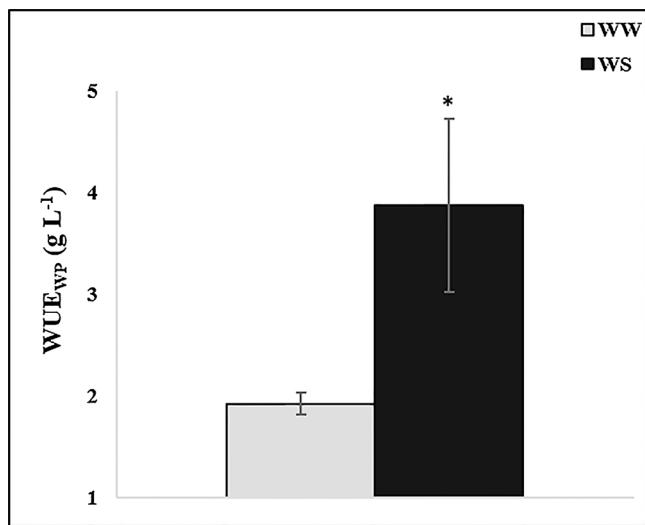
### 3.3. $g_{\text{night}}$ and $E_{\text{night}}$ drivers, implications and opportunities

Understand the mechanisms underlying the responses of  $g_{\text{night}}$  and  $E_{\text{night}}$  to water availability and the characterization of costs–benefits relationship can help to determine its causes and whether the occurrence and magnitude of those traits have or not acclimation value. In a previous study (Snyder et al., 2003), the magnitude of  $g_{\text{night}}$  was positively correlated with the magnitude of  $g_{\text{day}}$ , but in the present investigation, we observed an opposite pattern (Table 2), as  $g_{\text{night}}$  presented a significant negative correlation with  $g_{\text{day-mo}}$  ( $r = -0.941$ ;  $P < 0.001$ ) and  $g_{\text{day-mid}}$  ( $r = -0.926$ ;  $P < 0.001$ ), in line with the evidences of a separate genetic control of  $g_{\text{night}}$  and  $g_{\text{day}}$  (Caird et al., 2007; Christman et al., 2009). Similarly, some reports stated that daytime conditions favoring  $A_N$  resulted in higher  $g_{\text{night}}$  (Easlon and Richards, 2009), while in the present study (Table 2), we observed a negative correlation between  $g_{\text{night}}$  and  $A_{N\text{mo}}$  ( $r = -0.913$ ;  $P < 0.001$ ) and  $g_{\text{night}}$  and  $A_{N\text{mid}}$  ( $r = -0.911$ ;  $P < 0.001$ ) as in the study of Christman et al. (2009). Resco de Dios et al. (2015) also suggested that  $g_{\text{night}}$  is independent of  $A_N$ , since different levels of radiation did not affect  $g_{\text{night}}$ . Together, these data demonstrated that in olive tree the previous  $g_{\text{day}}$  and  $A_N$  do not determine following  $g_{\text{night}}$ .

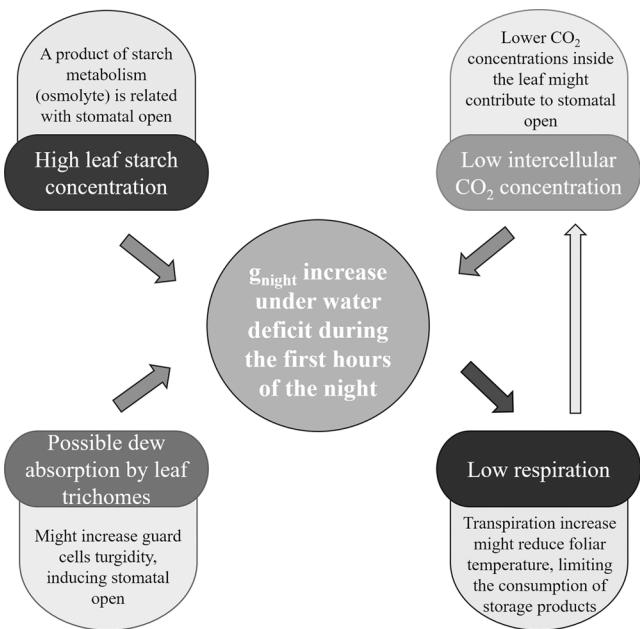
It was proposed that a by-product of starch-metabolism (osmoticant) may affect guard cells osmoregulation (Easlon and Richards, 2009), causing a great stomatal opening when St levels are high (Caird et al., 2007). Likewise, a positive correlation between  $g_{\text{night}}$  and St concentrations were found in the present study ( $r = 0.974$ ;  $P < 0.001$ ) (Tables 2 and 3). Meanwhile, higher  $E_{\text{night}}$  of WS plants can play a crucial role, once may lower leaf temperature by evaporative cooling, thereby decreasing carbon losses through R (Coupel-Ledru et al., 2016), an hypothesis supported by the significant negative correlation between  $E_{\text{night}}$  and  $R_{\text{night}}$  ( $r = -0.622$ ;  $P < 0.05$ ). This response takes especially importance in the first hours of darkness, once temperatures are usually higher. In addition, a reduced accumulation of Ci<sub>night</sub> (Table 2), due to the reduced  $R_{\text{night}}$  ( $r = 0.949$ ;  $P < 0.001$ ), may have contributed to stomatal open (Escalona et al., 2013), as attested by the negative correlation between Ci<sub>night</sub> and  $g_{\text{night}}$  ( $r = -0.734$ ;  $P < 0.001$ ). Furthermore, the possible dew absorption by trichomes (Munné-Bosch et al., 1999; Savé et al., 2000), may immediately increase foliar hydration and guard cells turgidity, what in turns could induce the higher  $g_{\text{night}}$  observed in this study. Moreover, this mechanism engineered by some plants to withstand drought stress could be suppressed under water availability, since is no longer needed (Munné-Bosch, 2010).

The substantial water cost of  $g_{\text{night}}$  could represent a major problem for agronomic development (Resco de Dios et al., 2015), reducing daily WUE, particularly under water deficit conditions (Escalona et al., 2013; Fuentes et al., 2014; Medrano et al., 2015). Nonetheless, in the present experiment the whole-plant WUE (Fig. 3), as well the  $A_{N\text{mid}}/g_{\text{day-mid}}$  (Table 2) were higher in WS than in WW plants. Likewise, higher WUE<sub>WP</sub> in drought stressed plants was reported previously in this olive tree genotype (Bacelar et al., 2007b).

In addition to evaporative cooling (Coupel-Ledru et al., 2016), the nutrient acquisition at root level and distribution within the plant has been proposed as a potential benefit of  $E_{\text{night}}$  (Scholz et al., 2007; Snyder et al., 2003, 2008). Drought is known to reduce both nutrient uptake capacity and availability (Kreuzwieser and Gessler, 2010). In the



**Fig. 3.** Whole-plant water use efficiency ( $\text{WUE}_{\text{WP}}$ ) of olive control plants (WW) and olive stressed plants (WS). Bars are means  $\pm$  SE ( $n = 8$ ). Significant differences: \* – significant at  $p < 0.05$ .



**Fig. 4.** Schematic representation relating leaf nighttime stomatal conductance ( $g_{\text{night}}$ ) with starch concentrations, intercellular  $\text{CO}_2$ , trichome layer and respiration rate.

present study, WS plants presented a lower production of biomass and substantial changes in concentrations, root uptake efficiency and physiological use efficiency of some mineral elements, being particularly more relevant the significant decrease of root uptake efficiency of phosphorus, potassium, sulfur and copper and the significant increase of phosphorus use efficiency, joining with slight increases of the physiological use efficiency of the other three elements (data not shown). Thus, altogether, these responses suggest that WS plants could increase nighttime transpiration in order to compensate the reduced mineral absorption capacity induced by drought stress.

Another potential benefit of  $E_{\text{night}}$  relies on the nocturnal foliar uptake of nutrients (Burkhardt and Hunsche, 2013), as the moisture accumulated by hygroscopic particles on dense trichome layers can favor the “hydraulic activation of stomata”, forming a continuous thin liquid water films on stomatal walls from external leaf surface to the

apoplast, allowing the flow of water and solutes driven by concentration differences (Burkhardt, 2010; Burkhardt et al., 2012). Nevertheless, if too many stomata are activated transpiration can highly increase and reduce drought tolerance of plants (Burkhardt, 2010; Pariyar et al., 2013).

#### 4. Conclusions

We were able to establish some correlations between some leaf traits and the nighttime transpiration response in the first hours of the night, as summarized in Fig. 4. However, in some cases when it starts the causes or the consequences of this response, as well as the costs and benefits, are still not clear. The present study revealed the increase of  $g_{\text{night}}$  in the first hours of the night, until certain level of water deficit could be coupled with some potential benefits to the plant. Nevertheless, the maintenance of  $E_{\text{night}}$  for longer periods of drought could have devastating consequences, leading us to believe that the potential benefits conferred by  $E_{\text{night}}$  might be dependent on stress severity, suggesting a threshold drought stress intensity for  $g_{\text{night}}$  that may be species dependent.

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